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In Vitro Preselection of Gene-Trapped Embryonic Stem Cell Clones for Characterizing Novel Developmentally Regulated Genes in the Mouse

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We have developed an *in vitro* gene trap screen for novel murine genes that allows one to determine, prior to making chimeric or transgenic animals, if these genes are expressed in one or more specific embryonic tissues. Totipotent embryonic stem (ES) cells are infected with a retroviral gene trap construct encoding a selectable *lacZ/neo*^{R} fusion gene, which is expressed only if the gene trap inserts within an active transcription unit. G418-resistant ES cell clones are induced to differentiate *in vitro*, and neurons, glia, myocytes, and chondrocytes are screened for expression of β-galactosidase (β-gal). cDNAs of the gene trap transcripts are obtained by 5′ rapid amplification of cDNA ends and are sequenced to determine if they represent novel genes. *In situ* hybridization analyses show that trapped genes are expressed *in vivo* within the cell types that express β-gal *in vitro*. Gene traps and their wild-type alleles are characterized in terms of copy number, alternate splicing of their transcripts, and the proportion of endogenous mRNA sequence that is replaced by *lacZ/neo*^{R} in the hybrid gene trap transcript. This approach, which we term "*in vitro* preselection," is more economical than standard *in vivo* gene trap screening because tissue-specific expression of probable knockout alleles is verified before transgenic animals are generated. These results also highlight the utility of ES cell differentiation *in vitro* as a method with which to study the molecular mechanisms regulating the specification and commitment of a variety of cell and tissue types.

INTRODUCTION

One of the fundamental problems of studying mammalian developmental genetics is that, unlike other model organisms (e.g., nematodes, flies, and frogs), the embryo cannot be readily observed as it develops. Thus, detecting phenotypic defects caused by mutations is difficult and is further complicated by *in utero* reabsorption of nonviable offspring. For this reason, "reverse genetic" methods (in which a gene is characterized before its mutant phenotype is known) have primarily been used to study the molecular basis of murine development. The two principal reverse genetic approaches both modify the genomes of embryonic stem (ES) cells, which are eventually introduced into blastocysts to secure germline transmission of mutant alleles (for reviews see Joyner, 1991; Hill and Wurst, 1993; Soriano, 1995). One approach, targeted or site-directed mutagenesis, was designed to disrupt the function of specific murine genes that are known by virtue of their homology to genes of other organisms. The principal drawback of this approach is that the sequence of the targeted gene must be known in order to carry out insertional or replacement mutagenesis (Bronson and Smithies, 1993; Soriano, 1995).

The other approach, gene trapping, was designed to identify novel, developmentally regulated genes in mouse embryos (Gossler *et al.*, 1989; Friedrich and Soriano, 1991; Skarnes *et al.*, 1992; Wurst *et al.*, 1995). Gene trap constructs contain the neomycin resistance gene (*neo*^{R}) and a promoterless *lacZ* (β-galactosidase or β-gal) reporter gene that has a splice acceptor consensus sequence at its 5′ end. These constructs are introduced by electroporation or retroviral infection into cultured ES cells, which are derived from the inner cell mass of blastocyst-stage embryos (Mar-

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tin, 1981; Evans and Kaufman, 1981). Colonies of ES cell clones containing the gene trap are selected by resistance to the antibiotic G418 and are expanded as undifferentiated cells *in vitro*. These cells can be injected into blastocysts, where they give rise to somatic and germline cells in the resulting chimeras (Bradley and Robertson, 1986). Expression of the gene trap *in vivo* is assayed by staining chimeric embryos with the β-gal substrate, X-gal. β-gal expression requires that the gene trap has inserted within a transcription unit, since the *lacZ* gene lacks its own promoter. Consequently, trapped genes transcribe an mRNA in which endogenous codons 3′ to the insertion site are replaced by *lacZ*. In addition to being mutagenic, the *lacZ* insertion facilitates cloning cDNAs of exon(s) upstream of the insertion site by 5′ RACE (5′ rapid amplification of cDNA ends) (Gossler and Zachgo, 1993; Chen, 1996).

In vivo gene trap screens allow for the identification of novel genes that are expressed either within specific tissues or in spatiotemporal patterns that may be of interest to the investigator (Gossler *et al.*, 1989; Friedrich and Soriano, 1991; Skarnes *et al.*, 1992; Wurst *et al.*, 1995). The developmental function of a given trapped gene may be elucidated by breeding transgenic animals that are heterozygous for the gene trap and analyzing homozygous offspring for morphological or physiological defects.

As an alternative to *in vivo* gene trap screens, which typically involve generating hundreds of chimeric embryos (Friedrich and Soriano, 1991; Wurst *et al.*, 1995), gene trap expression within specific cell types may be monitored in differentiated ES cell cultures. Although aggregates of differentiated ES cells, called embryoid bodies (EBs), do not exhibit normal morphogenesis, they give rise to a range of embryonic cell types (for reviews see Pederson, 1994; Keller, 1995). Approximately one in three EBs generates blood islands similar to those observed in the yolk sac (Doetschman *et al.*, 1985; Wiles and Keller, 1991). Angiogenesis can occur within EBs, and blood cells have been observed to "flow" through endothelial channels in the vicinity of beating foci of cardiomyocytes (Wang *et al.*, 1992). Both cardiac and skeletal myocytes spontaneously differentiate in EBs (Doetschman *et al.*, 1985; Miller-Hance *et al.*, 1993; Wobus *et al.*, 1994; reviewed in Baker and Lyons, 1996). Chondrogenesis has been observed to a lesser degree than myogenesis (Martin, 1981). When induced by retinoic acid, embryoid bodies generate neurons and glia (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Finley *et al.*, 1996). The proper temporal sequence of expression has been observed *in vitro* for neurogenic genes (Fraichard *et al.*, 1995), hematopoietic genes (reviewed in Keller, 1995), and myogenic genes (reviewed in Baker and Lyons, 1996).

ES cell cultures, therefore, provide a simple model system for studying the genetic pathways that regulate embryonic tissue development. Taking advantage of this system, we have developed a more economical approach, which we call "*in vitro* preselection," for identifying and characterizing novel genes that are not constitutively expressed but are expressed in selected cell types. (A schematic overview of *in vitro* preselection is presented in Fig. 1.) ES cells containing the ROSAβ-geo gene trap (Friedrich and Soriano, 1991) are allowed to differentiate *in vitro* in the absence of G418, to determine if limited cell types retain expression of the gene trap. Differentiated ES cells are assayed for coexpression of β-gal and antigens present specifically in neurons, glia, myocytes, or chondrocytes. To determine if the mutagenized gene expressed by these cell types is novel, a cDNA of the gene trap transcript is generated by 5′ RACE, cloned, and sequenced. Novel cDNAs are subjected to further molecular analyses to determine the relative likelihood that the gene-trapped allele will result in an overt phenotype in homozygous transgenic embryos. The criteria used are listed in order of importance: (1) the gene has a single copy per haploid genome; (2) the transcript encoded by the gene-trapped allele lacks most of the endogenous sequence; (3) the transcript is not alternately spliced; and (4) the endogenous gene is expressed in a restricted set of tissues *in vivo*. Using *in vitro* preselection, we have isolated four single-copy genes, each encoding a single transcript, that are expressed in the developing nervous system, heart, or limb cartilage. Three of these genes are novel, and one had been previously identified as a developmentally important gene by an *in vivo* gene trap screen. We are currently generating transgenic animals to investigate the developmental functions of the three novel genes.

MATERIALS AND METHODS

Embryonic Stem Cell Culture

The ES cell line R1 (kindly provided by Drs. Andras Nagy and Janet Rossant) was propagated in an undifferentiated state on feeder layers of STO fibroblasts (American Type Culture Collection) pretreated with mitomycin C (Sigma) according to Hogan *et al.* (1993). The growth medium consisted of 15% fetal bovine serum (FBS; Intergen or Hyclone), pretested for maintaining ES cells in an undifferentiated state, supplemented with 0.1 m*M* β-mercaptoethanol, 2 m*M* L-glutamine, 0.1 m*M* MEM nonessential amino acids, and 0.1% (v/v) leukemia inhibitory factor in DMEM (Doetschman *et al.*, 1985; Williams *et al.*, 1988). Growth medium was changed daily and ES cells were transferred to new feeder layers or allowed to differentiate (see below) when the colonies approached 70–80% confluence.

Gene Trap Construct and Retroviral Infection

The gene trap vector used in these experiments was ROSAβ-geo (Friedrich and Soriano, 1991). ROSAβ-geo was packaged by the GP + E86 cell line into viral particles (Soriano *et al.*, 1991), which were secreted into the surrounding buffalo rat liver (BRL) cell-conditioned medium (Smith and Hooper, 1987). This medium was filtered through a 0.2-μm filter and placed over undifferentiated R1 cells (plated at a density of 10^6 cells per 100-mm tissue culture dish). After 20 hr, the medium was replaced with fresh BRL-conditioned medium for 1 day. On the following day, infected cells were selected on BRL-conditioned medium containing the antibiotic G418 (400 μg/ml; Gibco/BRL) for 7–10 days. During this period, noninfected cells died and detached from the plate; infected cells generated colonies 7–10 days after infection. Colonies were picked

under a dissecting microscope, trypsinized to disaggregate the cells, and seeded onto duplicate multiwell dishes in growth medium containing STO cells and 200 μg/ml G418. All G418-resistant ES cell clones were divided into multiple aliquots in freezing medium (10% DMSO in growth medium) and stored at −80°C or under liquid nitrogen.

In Vitro Differentiation of ES Cells

ES cells were dissociated from feeder layers by adding 0.05% trypsin–EDTA for 1 min, and STO cells were removed by preplating the STO/ES cell mixture onto tissue culture plates once for 15–60 min. The ES cells were cultured in suspension (Robertson, 1987) in petri dishes in differentiation medium (10% FBS, 2 m*M* L-glutamine, 0.1 m*M* MEM nonessential amino acids in DMEM; Gibco/BRL), which was changed daily. EBs spontaneously differentiated and were plated onto tissue culture dishes (for X-gal staining) or gelatinized coverslips (for immunolabeling) after 6–8 days of suspension when endoderm and ectoderm appeared in >50% of the EBs. Beating foci of cardiomyocytes appeared 4–7 days after plating.

To enhance neuronal development, some EBs were treated with 0.5 μ*M* all-*trans* retinoic acid (Sigma) added to differentiation medium with 15% FBS between Days 4 and 8 (Bain *et al.*, 1995; Finley *et al.*, 1996). The embryoid bodies, either undissociated or dissociated in trypsin for 10 min, were seeded onto gelatin-coated coverslips. After 2 days, the cultures were treated with 10 μ*M* cytosine arabinoside (Sigma) to inhibit further division of undifferentiated cells.

For chondrocyte development, 10% calf serum (Intergen) was substituted for FBS and the medium contained 1 μ*M* dexamethasone (Poliard *et al.*, 1995). Coverslips were coated with rat tail collagen I (10 mg/ml in PBS) before plating embryoid bodies. Chondrogenic foci that stained with 1% Alcian blue 8GX in 0.1 *N* HCl (Lev and Spicer, 1964) appeared in small numbers after 1–2 weeks interspersed with other cell types.

Histochemistry and Immunofluorescence

Differentiated EBs plated on tissue culture plastic for X-gal staining were examined daily on an inverted phase-contrast microscope for beating cardiomyocytes, skeletal myotubes, chondrogenic foci, or clusters of neurons. The positions of muscle cells were recorded for future reference by carefully drawing a circle around the contracting or beating cells on the bottom of the dish. These cultures were rinsed twice in PBS, fixed with 2% formaldehyde in PBS at room temperature for 5 min, and rinsed again in PBS. The cells were stained at 37°C for 4 hr or overnight in X-gal staining solution according to Hogan *et al.* (1994) and analyzed under brightfield and phase-contrast microscopy. Cell lines in which putative neurons, skeletal myocytes, or cardiomyocytes stained with X-gal were further examined by immunofluorescence.

Differentiated EBs plated onto gelatinized coverslips for immunolabeling were fixed in fresh 4% paraformaldehyde at 37°C for 15 min, rinsed in PBS, and postextracted in 0.1% Triton X-100 for 6 min. For immunofluorescent labeling of chondrocytes, fixed cultures were treated with 0.6 mg/ml bovine testicular hyaluronidase (440 U/mg, Sigma) in 160 m*M* sodium phosphate, pH 5.9, 39 m*M* NaCl for 2 hr before the postextraction. All cultures were incubated for 2 hr at room temperature in blocking solution (5% BSA, 2% normal goat serum, and 0.1% Triton X-100 in PBS; Haendel *et al.*, 1996) and incubated overnight at 4°C in blocking solution containing one or more primary antibodies diluted as follows: 1:3000 rabbit anti-β-gal (Cappel) or 1:500 mouse anti-β-gal (Gibco/BRL); 1:1000 mouse anti-βIII-tubulin (provided by Dr. Anthony Frankfurter); 1:250 rabbit anti-GFAP (Dako); 1:4 rat 401 (anti-nestin), 1:5 MF20 (anti-muscle myosin), or 1:50 CIIC1 (anti-collagen II). The last three antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The cultures were rinsed with PBS, incubated for 1 hr at 37°C with fluorescein- and rhodamine-conjugated secondary antibodies (1:200 or 1:1000 in blocking solution) specific for either mouse or rabbit IgG (Jackson ImmunoResearch), and rinsed several times in PBS.

To minimize photobleaching, samples were mounted in 90% glycerol and 10% PBS containing 100 μg/ml DABCO (Calbiochem) and 1 μg/ml *p*-phenylenediamine (Sigma). Immunolabeled EBs were initially analyzed with a Zeiss Axiophot microscope equipped with epifluorescence. Double-labeled micrographs were generated with a Bio-Rad 1000 confocal microscope, and intracellular coexpression of antigens was indicated by a yellow color resulting from the overlapping red (rhodamine) and green (fluorescein) signals. Control cultures treated with only secondary antibodies showed no cell-specific labeling (Fig. 2B).

Cloning Gene Trap Transcripts by 5′ RACE

Infected ES cell lines that expressed *lacZ* in neurons, muscle, or chondrocytes were propagated as undifferentiated cells. RNA was harvested from these cells with Trizol (Gibco/BRL), and the polyadenylated fraction was isolated with the PolyATtract kit (Promega). A 25-base pair (bp) primer that binds *lacZ* was used for primer extension. Forty picomoles of a *lacZ*-specific primer (2629: 5′-CCG TGC ATC TGC CAG TTT GAG GGG A-3′; this primer binds β-*geo* 239 base pairs downstream of the β-galactosidase ATG) and 5–10 μg of poly(A)$^+$ RNA at 60°C for 20 min in the presence of RNasin (Promega) were used. The primed RNA was cooled on ice, and cDNA was generated by adding a reaction cocktail with a final composition of MuLRV reverse transcriptase (RT; Boehringer-Mannheim), 1× RT reaction buffer, 0.65 μ*M* each dNTP, 2.5 m*M* DTT, and 30 m*M* spermidine. Primer extension was allowed to proceed for 2 hr at 37°C. The RNA was then degraded by addition of 1 μg DNase-free RNase for an additional 15 min at 37°C. dH_2O (300 μl) was added to the primer extension product, and buffer salts, dNTPs, and primers were eluted through a Millipore Ultrafree 30,000 NMWL filter. A polyA tail was added to the recovered cDNA with 125 units of terminal deoxynucleotidyl transferase (TdT; Boehringer-Mannheim) and 125 pmol dATP for 5 min at 37°C. The TdT was inactivated by heating the mixture to 65°C for 5 min.

A second cDNA strand was primed from an oligonucleotide containing a PCR anchor and 18 tandem thymidine residues (PTCor: 5′-CGA GGG GGA TGG TCG ACG GAA GCG ACC T_{18}-3′). The double-stranded cDNA was amplified using a primer specific to the anchor (9404: 5′-GAT GGT CGA CGG AAG CGA-3′) and a primer downstream of the splice acceptor sequence of the ROSAβ-geo vector (M2: 5′-GTG CCG GAA ACC AGG CAA A-3′; this primer binds β-*geo* 160 base pairs upstream of the β-galactosidase ATG). PCR was performed in a Perkin–Elmer thermal cycler (25 cycles of 1 min at 94°C, 2 min at 64°C, 3 min at 72°C). One-tenth of the PCR volume was run on a 1% agarose minigel, Southern blotted and probed with a 5′ *lacZ*-specific probe to determine the size of the trapped cDNA. The remainder of the PCR product was run on an identical gel. DNA of the appropriate size was extracted from the gel, ligated into pCRII (Invitrogen) or pGEM-T (Promega), and transformed into competent JM109 cells (Promega). As a diag-

nostic for gene trap-specific sequences, transformed colonies were lifted onto a nylon membrane, lysed, and hybridized to a radiolabeled probe for the splice acceptor sequence (SAS) of ROSAβ-geo.

Sequencing and cDNA Library Screens

To determine if the trapped genes had been previously characterized, cDNAs were sequenced with the Sequenase kit (Amersham/USB) from a primer 70 base pairs downstream of the SAS. The obtained sequence was analyzed to confirm the presence of the SAS and a poly(A)$^+$ tract adjacent to the anchor sequence. The sequence between the SAS and poly(A)$^+$ tract (corresponding to part of the mutagenized gene) was compared with sequences contained in the GenBank and EMBL databases.

For trapped genes that had not been previously characterized, we screened two libraries for homologous full-length cDNAs: a λgt10 library of cDNAs from E8.5 embryos (kindly provided by Dr. Brigid Hogan) and a λgt11 library of cDNAs from differentiated ES cells (kindly provided by Dr. Jeffrey Robbins). Library plating and screening were carried out by standard methods (Sambrook *et al.*, 1989). We obtained one or more larger cDNAs of the endogenous 6AC6, 8AB2, and 17BC4 transcripts. The sequences of these larger cDNAs confirmed that these genes have not been previously identified.

The largest 8AB2 cDNA (from the λgt11 library) we obtained was 2.95 kb long. Both a 625-bp *Eco*RI fragment of this cDNA (containing the 3′ polyadenylation signal) and the 88-bp 5′ RACE product were used as templates for making radiolabeled probes for *in situ* hybridizations and Southern and Northern blot analyses. The largest 17BC4 cDNA obtained was 2.6 kb long. Two separate *Eco*RI fragments, 750 and 900 bp, were used as templates for making radiolabeled probes for *in situ* hybridizations and Southern blot Northern blot analyses. The largest 6AC6 cDNA was 1.65 kb long. Both a 600-bp *Eco*RI fragment of this cDNA and the 5′ RACE product were used as templates for making radiolabeled probes for *in situ* hybridizations and Southern and Northern blot analyses.

In Situ Hybridization and Molecular Analyses of Trapped Genes

Embryos were considered to be at embryonic stage E0.5 (i.e., 0.5 day postcoitum) on the morning the vaginal plug was observed. Tissue preparation and *in situ* hybridization were performed as previously described (Lyons *et al.*, 1996). cDNAs derived from 5′ RACE and from the cDNA libraries were used as templates for generating [^{35}S]UTP-labeled antisense cRNA probes for endogenous transcripts ([^{35}S]CTP was used for GC-rich templates). A 680-bp myf-6 (MRF4) gene-specific probe (Bober *et al.*, 1991) was used to colocalize expression of 8AB2 in embryonic skeletal muscle (Fig. 4). Sense control cRNA probes to 6AC6, 6BD4, 8AB2, 17BC4, and myf-6 showed only background levels of hybridization signal. The antisense and sense probes transcribed from gene trap constructs showed slightly higher background compared to those from cDNAs cloned from libraries due to the short polyA stretch at the 5′ end of the gene trap.

Copy numbers of the mutagenized genes were determined by genomic Southern blot analysis (Sambrook *et al.*, 1989), using both 5′ probes (derived from 5′ RACE) and 3′ probes (obtained from the λgt10 or λgt11 cDNA libraries). The sizes of wild-type and mutant transcripts were determined by standard Northern blot analyses (Sambrook *et al.*, 1989) of mRNAs derived from uninfected R1 cells and from the appropriate ROSAβ-geo-infected ES cell clones.

RESULTS

Differentiation and Histochemical Screening of ES Cells

We have infected undifferentiated ES cells (line R1) with the gene trap ROSAβ-geo vector (Friedrich and Soriano, 1991). This construct contains an RNA splice acceptor consensus sequence upstream of a promoterless β-galactosidase/neomycin resistance fusion gene (β-*geo*; see Fig. 1). Undifferentiated cell clones that express the gene trap were isolated as neomycin-resistant colonies and cultured on feeder layers. Each ES cell clone was assayed for β-gal expression by X-gal histochemical staining (Fig. 2A). As expected, most or all cells in undifferentiated colonies are stained by this procedure.

We cultured EBs under conditions that promote differentiation of neurons, myocytes, or chondrocytes and examined their developmental progress by phase microscopy. Some neurons and chondrocytes could be identified by morphological criteria. Distinctive networks of phase-dense neurons often formed at the periphery of plated EBs treated with retinoic acid (Bain *et al.*, 1995). Chondrocytes were initially identified by their characteristic aggregation into nodules and staining by Alcian blue (Ahrens *et al.*, 1977). Cardiomyocytes could be reliably identified by their rhythmic contractions. Skeletal muscle cells were identified by their slow contractions and their multinucleated morphology.

We initially stained differentiated EBs with X-gal to rapidly determine if gene trap expression is constitutive or selectively maintained in myocytes (Fig. 2B), chondrocytes, or neurons. Due to the heterogeneous nature of these cultures, it was not always possible to determine if the staining was within the cell types of interest. Nevertheless, this technique allowed us to determine which clones clearly did not express β-gal in the cell types we were interested in studying and indicated which clones deserved further study with immunolabeling techniques.

Eighty-six ROSAβ-geo-containing cell clones were differentiated *in vitro* and assayed for gene trap expression by X-gal staining. Seven days after the EBs were plated, 23% of the clones exhibited uniform β-gal expression, 22% exhibited no expression, and 55% expressed β-gal in limited cell types. Among the last group of clones, the majority did not exhibit β-gal expression within the cell types we screened (neurons, glia, chondrocytes, and muscle). For example, 8 of 45 cell lines that developed multiple foci of cardiomyocytes appeared to have X-gal staining in these foci (e.g., Fig. 2B), and 2 of 17 lines tested exhibited X-gal staining in neurons. These observations suggest that most genes trapped by ROSAβ-geo are expressed in a fashion that is restricted to certain cell types during the course of development, in agreement with the *in vivo* expression patterns reported previously (Friedrich and Soriano, 1991) and in this study.

Immunofluorescent Identification of Gene Trap-Expressing Cell Types

Immunofluorescent double labeling of differentiated EBs allowed us to unequivocally identify specific types of cells

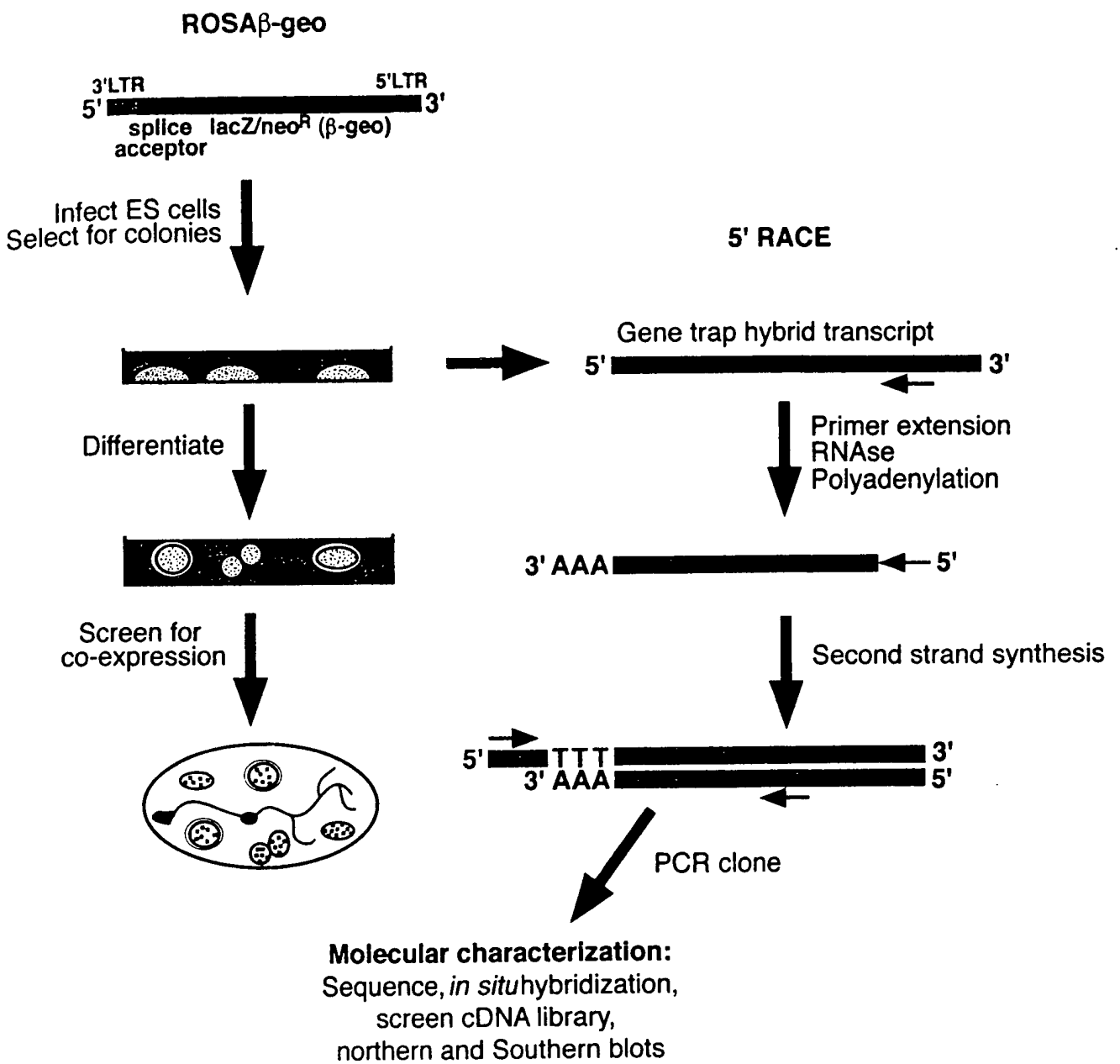


FIG. 1. Summary of the "*in vitro* preselection" procedure. Screening: ES cells are infected with the ROSAβ-geo retroviral vector. Retroviral LTRs are in reverse orientation to the β-geo sequence. The cells are allowed to differentiate *in vitro* and each clone is screened for maintained expression of the construct within cell types of interest, such as neurons. The selected clones are grown as undifferentiated cells and their poly(A)$^+$ RNA isolated. 5' RACE: The gene trap transcript is reverse transcribed from a *lacZ*-specific primer (see Materials and Methods) and the resulting first-strand cDNA is polyadenylated by terminal transferase. After RNA degradation, an anchor-polyT primer is annealed to the 5' end and the second strand generated. The cDNA is amplified by PCR using primers specific to the anchor and the splice acceptor. The products are visualized on a Southern blot using a probe specific to *lacZ*. The cDNAs are cloned into a commercial vector that accepts PCR fragments. Molecular characterization: The cDNAs are first sequenced to determine any homologies and then used as templates to generate cRNA probes for *in situ* hybridization analysis and cDNA probes for Northern and genomic Southern blotting and cDNA library screening. Candidates for a knockout gene trapped allele are chosen on the basis of their expression patterns, number of transcripts, amount of endogenous sequence remaining, and copy number.

and determine if they expressed β-gal. Positive and negative control experiments were conducted to show the sensitivity of anti-β-gal antibody (Fig. 3A) and the levels of nonspecific background with secondary antibodies alone (Fig. 3B). Skeletal myoblasts, neuroblasts, and glia express the intermediate filament nestin (Lendahl *et al.*, 1990; Sejersen and Len-

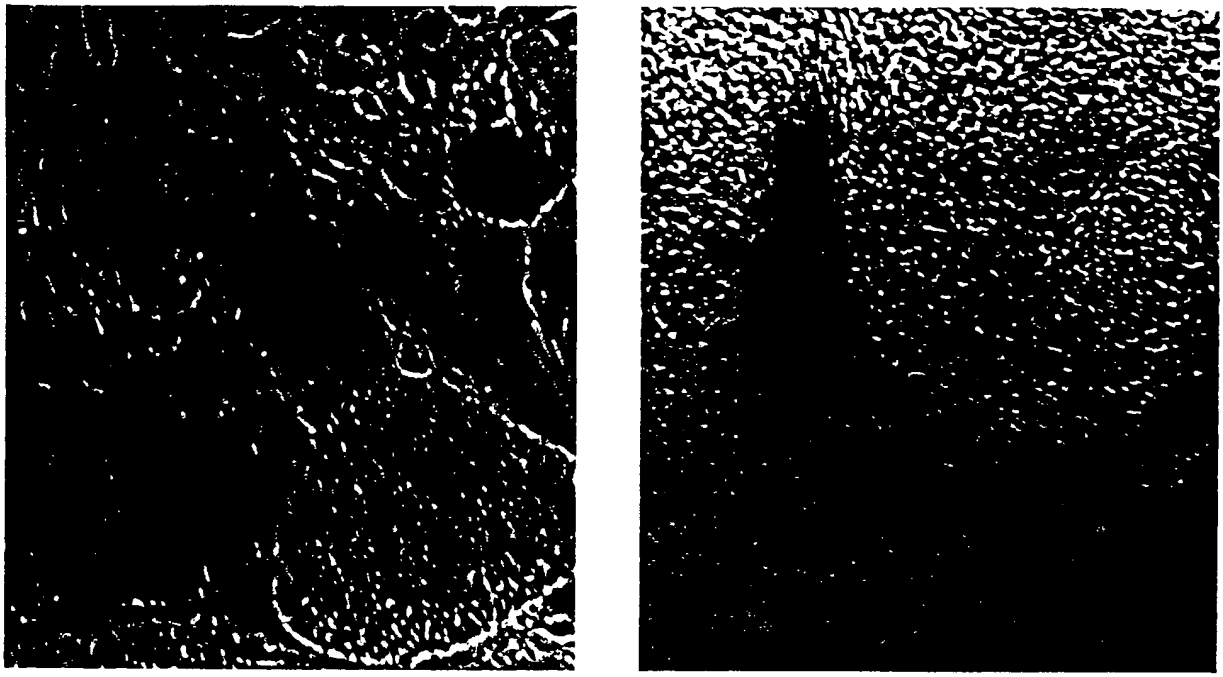


FIG. 2. Gene-trapped ES cells stained with X-gal before and after *in vitro* differentiation. (A) Undifferentiated colonies (arrowheads) and a differentiating colony (arrow) of ES cells derived from line 6BD4 were fixed and stained on a feeder layer of STO cells. All cells in the undifferentiated colonies express β-gal, whereas many cells have shut-off β-gal expression in the differentiating colony. (B) Differentiated 6BD4 cells derived from a single embryoid body plated 7 days earlier. Two foci of beating cardiomyocytes were observed, and their positions were carefully recorded by marking the bottom of the tissue culture dish with a fine-tipped pen. After fixing and staining the culture, both foci (and two others on the dish) exhibited heavy staining, suggesting that the 6BD4 gene trap was expressed in cardiomyocytes. This was confirmed by later experiments (see Figs. 3D and 5C). Scale bar = 100 μm.

dahl, 1993). Antinestin labeling permitted the identification of clones that express β-gal in one or more of these lineages at early stages of differentiation. Nestin and β-gal were coexpressed in multinucleated skeletal myotubes derived from clone 8AB2 (Fig. 3C). Using antibodies to sarcomeric myosin (MF-20; Bader *et al.*, 1982) and β-gal, we confirmed that clone 6BD4, which exhibited X-gal-stained foci of cardiac muscle (Fig. 2B), expresses the gene trap in cardiomyocytes (Fig. 3D). Coexpression of β-gal and the cartilage-specific collagen type II (George-Weinstein *et al.*, 1994) was observed for two ES cell clones; one of them, 6AC6, is shown here (Fig. 3E).

Neurons were identified with an antibody against βIII tubulin, which is expressed specifically in neurons (Memberg and Hall, 1995). Figures 4A–4D show two clones, 17BC4 and 8AB2, that maintain high levels of β-gal expression in neurons. After 2 weeks of differentiation, dispersed 17BC4 ES cells express β-gal at higher levels in mature neurons than in other cell types, with the exception of undifferentiated ES cells (Figs. 4A and 4B). The 8AB2 clone maintained expression of β-gal in a subset of neurons in 2-day-old dispersed cultures (Figs. 4C and 4D); however, coexpression of β-gal and βIII tubulin was not observed in 2-week cultures. Additionally, 8AB2 cells expressed β-gal in a subset of glia (Figs. 4E and 4F), as indicated by its intracellular colocalization with glial fibrillary acidic protein, GFAP (Fraichard *et al.*, 1995).

Four of these clones (6AC6, 6BD4, 8AB2, and 17BC4) were selected for molecular analyses of their trapped genes. Although all four cell lines express ROSAβ-geo in one or more distinctive differentiated cell types, each line also expressed the gene trap in cells that were not distinguishable by antigenic or morphological criteria. Given the heterogeneous nature of differentiated ES cell cultures, a proportion of cells labeled with the β-gal antibody were probably undifferentiated since we had originally selected cell lines by growing undifferentiated cells in the presence of G418. However, an undetermined proportion of these β-gal-labeled cells may represent differentiated cell types other than those for which we assayed.

Sequence Identification

We isolated RNA from approximately 1×10^8 undifferentiated cells in which the gene trap is highly expressed. After enriching for the poly(A)$^+$ RNA fraction, we used a *lacZ*-specific primer to perform 5′ RACE (5′ rapid amplification of cDNA ends), resulting in cDNAs corresponding to the gene trap transcripts (summarized in Fig. 1). We sequenced these cDNAs and compared them to gene sequence databanks to

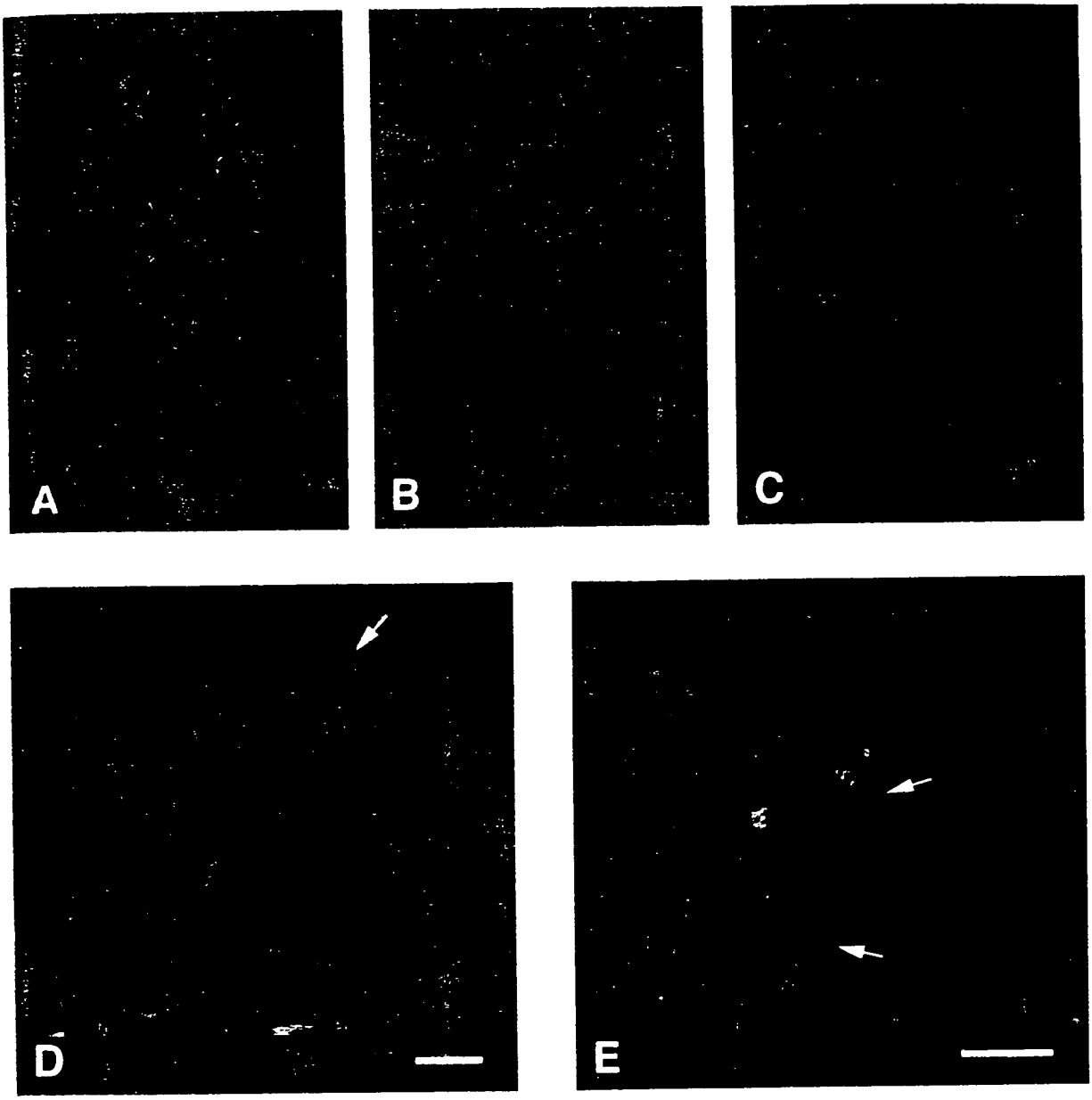


FIG. 3. Immunofluorescent double labeling of β-gal (fluorescein, green) and cell type-specific antigens (rhodamine, red) in cultured ES cells. (A) A differentiating colony of clone 6BD4 cells exhibits strong β-gal label in some cells but no label in others (compare with the X-gal stained colony in Fig. 2A). The colony was fixed 1 day after removal from the feeder layer. (B) A negative control showing cells from the 6BD4 clone labeled with both secondary antibodies only. (C) A multinucleated skeletal myocyte derived from clone 8AB2 shows colocalization of β-gal and nestin. Three nuclei appear as unlabeled round structures in the center of the cell. (D) A focus of cardiomyocytes from clone 6BD4 includes a cell (arrow) that expresses both β-gal and myosin heavy chain. (E) A nodule of chondrocytes (arrows) from clone 6AC6 coexpresses β-gal and intracellular type II collagen precursors. Cells shown in B–D were fixed 2 days after plating; cells in E were fixed 15 days after plating. Scale bars = 50 μm (A, B, and E) and 25 μm (C and D).

determine whether or not the trapped sequences are homologous to previously identified genes (Table 1). The gene designated *6BD4* was found to be identical to *jumonji*, which had been identified in an earlier gene trap screen (Takeuchi *et al.*, 1995); the other trapped gene sequences are novel.

Gene trap insertion results in a hybrid transcript consisting of a truncated endogenous sequence fused to β-geo. Consequently, the 5′ RACE products represent exon sequences upstream of the insertion site. Retroviral vectors tend to integrate near the 5′ end of genes (Friedrich and Soriano, 1991), so that relatively short (72–207 bp) sequences were obtained by 5′ RACE. Since these sequences

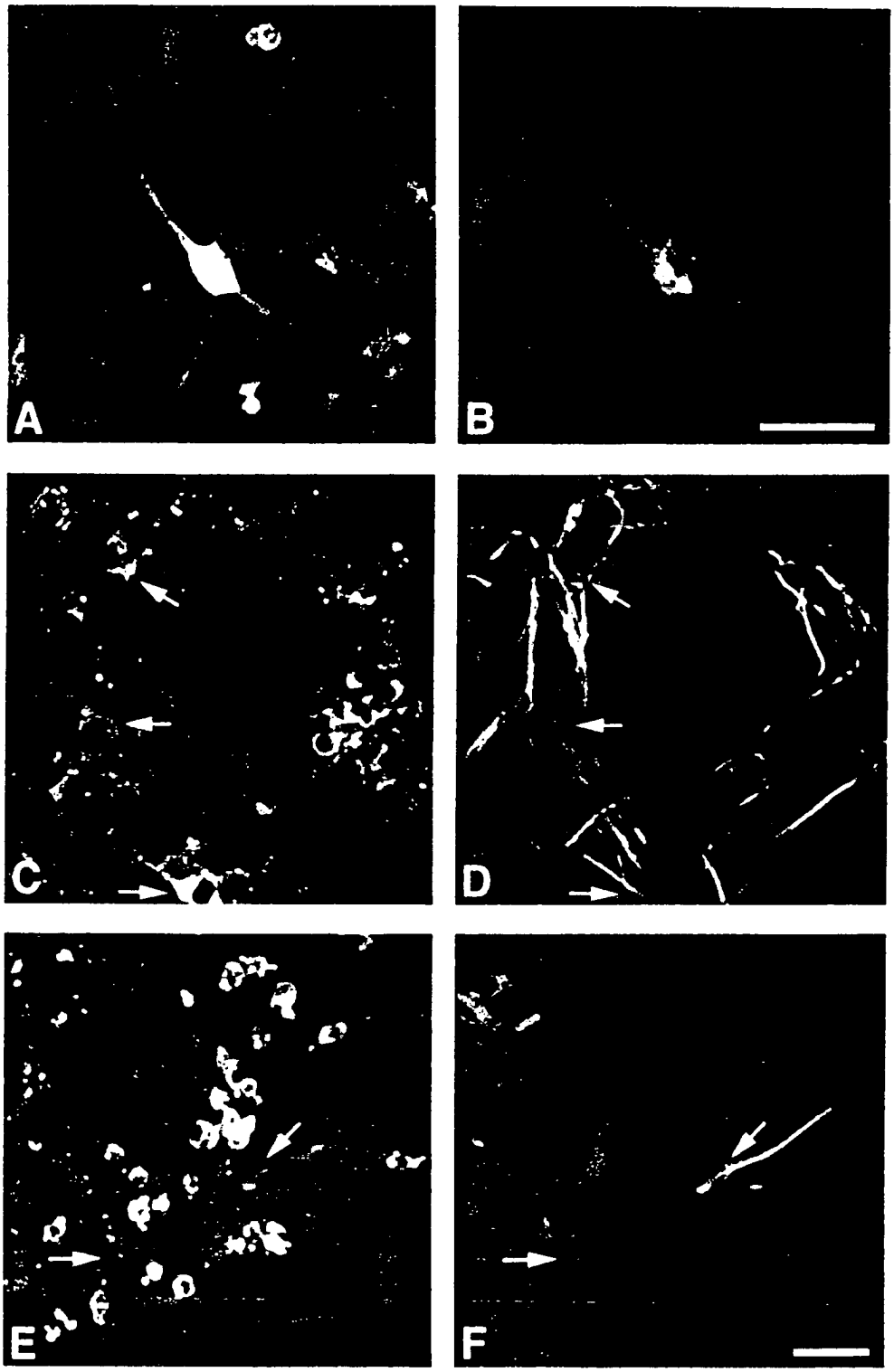


FIG. 4. Immunofluorescent double labeling of β-gal (A, C, E) and nervous system specific markers (B, D, F) in ES cell clones cultured under conditions that promote neuronal differentiation. (A, B) 17BC4 maintains a high level of β-gal expression (A) in a neuron labeled with βIII-tubulin (B). (C, D) A differentiated culture of 8AB2 cells exhibits β-gal expression (C) within the cell bodies of a subset of neurons (arrows), which were identified by their coexpression of βIII-tubulin (D). (E, F) Differentiated 8AB2 cells exhibit β-gal expression (E) within two immature glial cells (arrows), which were identified by GFAP labeling (F). Cells shown in A and B were fixed 7 days after plating; cells shown in C–F were fixed 2 days after plating. Scale bars = 50 μm.

TABLE 1
Sequences of Gene Trap cDNAs

Gene trap	Sequence
6AC6	TTTTTagccaccgctaccgccgccgcctcccgggtgggcgcccttctccttggacgccggcgacccaggacgagggcttcatcactg taaatggttgcaagccgacaaagctgcacctcctgaaaaagacggacagcccatcgcgtgagctgtagagtttgtggacgcattt ctatcgctcgcggttg
6BD4 (jumonji)	TTTTTgtatttcttctgaatgatattcctcttgggtctttccttgctcattctgagatccaaatggattgcaaaaggggacaattgc ttcatgaatttaaagccgtcagaaatttgcaaaaaaatatcctggtggtttttattgttgttggtggtggttgatcttgtaatccg actttttcttcctcttcccccaaaaccagattcgcctgctcgcggttg
8AB2	TTTTTggggtctcctcagcggccggctggagtcggtacgtccttgcccgagctcatcagccgaggcgtgtggcagcaaaccccagct ccaacctcgcggttg
17BC4	TTTTTtgaggaaggcggcggcggtgtggctgcggcgagcgcgacactccctgcagcggagtgctcggtggaagaggctcgcggttg

Note. Partial cDNAs of four ROSAβ-geo-trapped transcripts (*6AC6*, *6BD4*, *8AB2*, and *17BC4*) were generated by 5′ RACE. The sense strand sequences for the endogeneous 5′ exon(s) are presented. All endogenous sequences are flanked by a 5′ polyTtract (represented by uppercase T's), which corresponds to the polyA tail that we added onto the first strand (antisense) primer extension product. The boldfaced sequence at the 3′ end (**ctcgcggttg**) is part of the ROSAβ-geo vector at the splice acceptor junction (Friedrich and Soriano, 1991).

may correspond only to 5′ untranslated regions (UTRs), they were used to generate probes for screening an E8.5 embryonic cDNA library (Fahrner *et al.*, 1987) and a differentiated ES cell cDNA library (Robbins *et al.*, 1990). In this manner we obtained more complete sequences of the endogenous genes (which will be described elsewhere). The large cDNA clones were sequenced to confirm that *6AC6*, *8AB2*, and *17BC4* have not been previously identified and were used as templates for *in situ* hybridization analyses and Southern blot analyses (see below).

In Situ Hybridization Analyses

Previous studies have shown that gene expression in differentiating ES cells *in vitro* recapitulates the developmental sequence of expression seen in embryos *in vivo* (reviewed in Pederson, 1994; Keller, 1995; Baker and Lyons, 1996). Therefore, we expected that novel genes identified by gene trapping would be expressed within the same cell types *in vitro* and *in vivo*. We analyzed the transcription patterns of endogenous genes by *in situ* hybridization of radiolabeled probes to a developmental series of sectioned mouse embryos. Most of the genes we examined exhibited widespread expression at E6.5, the time at which the primary germ layers form and tissue morphogenesis begins. Given that each ES cell clone was selected on the basis of β-geo expression in undifferentiated cells, it was not surprising that early embryonic expression was widespread. Expression of all of the genes became more restricted at later stages of development, although the degree of down-regulation varied considerably between genes.

Each gene selected from the *in vitro* screen was expressed within the tissues predicted by ROSAβ-geo expression differentiated ES cells (Fig. 5). Gene expression was not limited to these predicted tissues, however, consistent with our observations of gene trap expression *in vitro*. Of the four genes we studied in detail, *6AC6* (identified in chondrocytes *in vitro*) showed the most limited expression pattern at later developmental stages. *6AC6* expression was restricted to the base of the developing limbs by E10.5, and by E11.5 expression was observed only in the chondrocytes of the shoulder or pelvic girdle (Fig. 5A). *6AC6* expression remained confined to the developing limbs between stages E12.5 and E15.5 and was not detected at later stages (not shown). Figure 4B shows a *6AC6* sense probe control to illustrate the background level of *in situ* hybridization signal with a probe generated from a 5′ RACE product. Expression of *6BD4* (identified in cardiomyocytes *in vitro*), which showed widespread expression at E8.5, exhibited more limited expression at E9.5 with the most prominent expression in the neuroepithelium and outflow tract of the heart (Fig. 5C), consistent with previously reported observations (Takeuchi *et al.*, 1995). The two other genes, *8AB2* and *17BC4*, maintained widespread expression during most of development. *8AB2* (identified in skeletal myocytes, neurons, and glia *in vitro*) expression was most prominent in the nervous system by E11.5 (Fig. 5D). We have not yet ascertained what proportion of this embryonic expression occurs in neurons vs glia. *8AB2* expression was detected in developing skeletal muscle (Fig. 5E) in E13.5–E16.5 embryos. To aid in the identification of cells that expressed the gene trap, we frequently used tissue-specific cRNA probes on sections consecutive to those hybridized with gene trap probes. Figure 5F shows a section serial to the one in Fig. 5E hybridized with a probe to *myf-6* (Bober *et al.*, 1991), which is a myogenic regulatory factor mRNA specific for skeletal muscle. *17BC4* (identified in neurons *in vitro*) maintained widespread expression until E15.5, when it was expressed at relatively higher levels in the nervous system. Neuronal expression continued into adulthood and in the brain was most prominent in the granule layers of the cerebellum (not shown) and hippocampus (Fig. 4G). Figure 5H shows a section consecutive to that in Fig. 4G which was hybridized to the 17BC4 sense control cRNA probe. The sense and antisense probes to *17BC4* were generated from a cDNA cloned from a differentiated ES cell library.

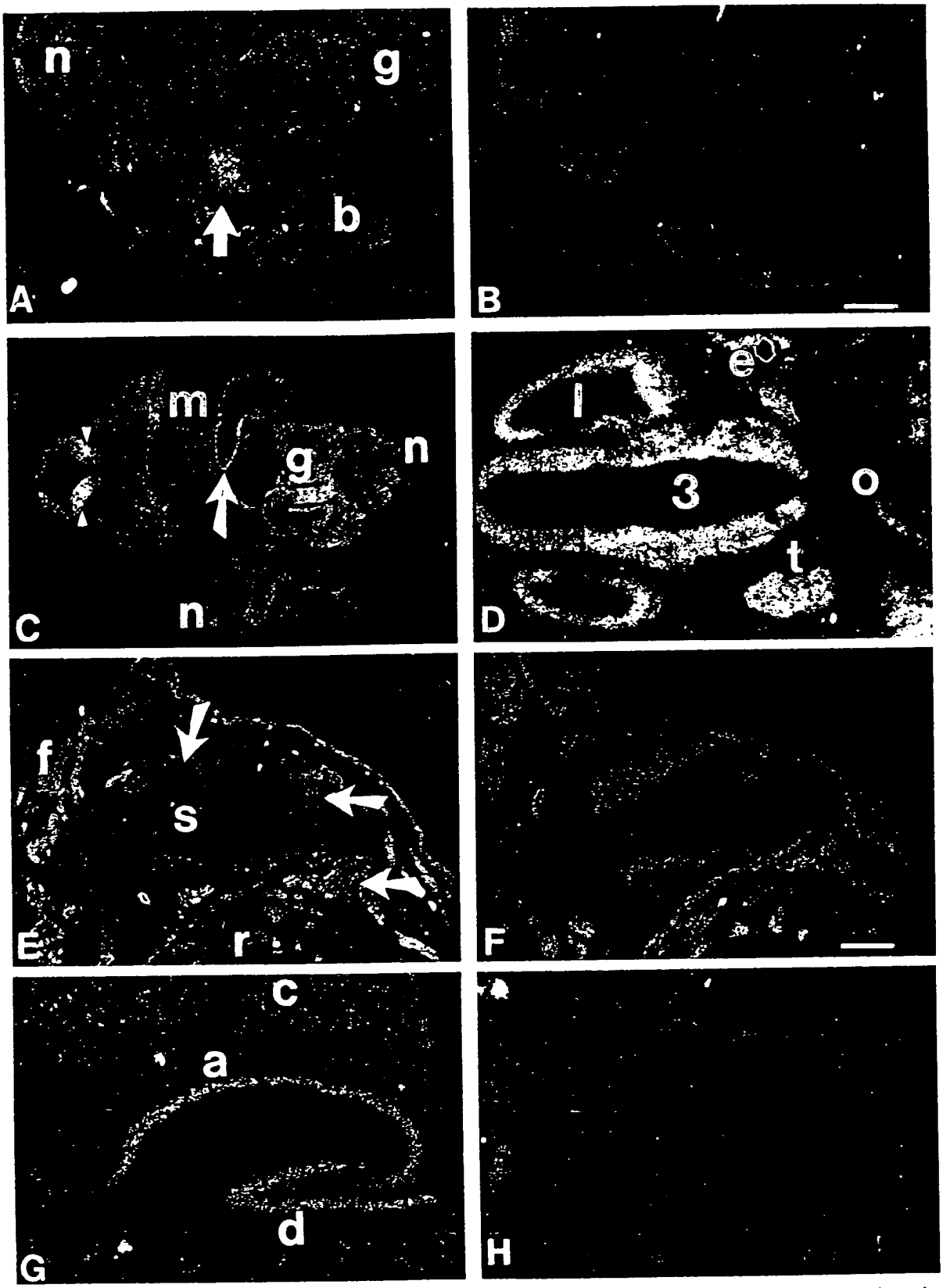
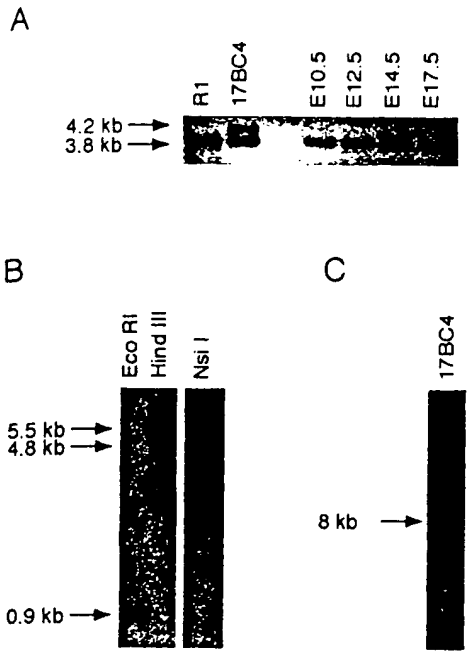


FIG. 5. Expression of endogenous transcripts in mouse tissues. (A, B) Darkfield micrographs of transverse sections through an E11.5 embryo hybridized with the antisense probe, generated from the 5′ RACE product (A) and the sense control probe (B) for *6AC6*. Arrow in



G. 6. Northern and Southern blot analyses. (A) Northern blot RNA derived from R1 cells, 17BC4 ES cell clone, and whole bryos. One microgram of poly(A)$^+$ RNA fraction was loaded in h lane, and the blot was hybridized sequentially to probes spe- ic for *17BC4* and *lacZ* sequences. The endogenous *17BC4* tran- ipt (3.8 kb) is labeled in both lanes, whereas the hybrid gene trap nscript (4.2 kb) is detected only within the lane from the clone taining ROSAβ-geo. One microgram of poly(A)$^+$ RNA extracted m a developmental series of whole embryos was loaded in each e. A single *17BC4* gene transcript is detected at each embryonic ge. (B) Southern blot of mouse genomic DNA (10 μg per lane) ested with *Eco*RI, *Hin*dIII, or *Nsi*I, blotted, and hybridized with *7BC4*-specific probe. A single band was detected in each lane, icating that the gene is a single copy and has no close homologs. Southern blot of genomic DNA isolated from the 17BC4 cell e. Ten micrograms of DNA was digested with *Eco*RI, blotted, hybridized with a probe specific to the 5′ end of β-*geo*.

rthern and Southern Analyses

urther molecular characterizations of each trapped gene its transcript allowed us to predict if the insertional mutation removed most or all of the coding region and thus was likely to produce a developmental phenotype. An example of such an analysis is presented for clone 17BC4 (Fig. 6). To determine if the endogenous transcripts had splice variants or were members of a multigene family, we looked for the presence of multiple bands on Northern blots made from RNA derived from R1 cells. We also blotted RNAs from infected ES cell lines to determine how much endogenous sequence was deleted due to insertion of the gene trap. Figure 6A shows a Northern blot probed with a 750-bp cDNA corresponding to a 5′ portion of the *17BC4* RNA. A single 3.8-kbp band is present in the R1 lane, representing the endogenous mRNA, which apparently is not alternately spliced. In the 17BC4 lane, representing RNA derived from the heterozygous 17BC4 cell line, there is an additional 4.2-kbp band corresponding to the hybrid gene trap transcript. Since the coding sequence for β-*geo* is 3.9 kbp (Friedrich and Soriano, 1991), the hybrid transcript retains approximately 300 bp of endogenous *17BC4* sequence. Therefore, the hybrid RNA probably encodes a protein devoid of normal function. On this same Northern blot, a single *17BC4* mRNA is detected in 1 μg of poly(A)$^+$ RNA extracted from whole embryos at E10.5, E12.5, E14.5, and E17.5 (Fig. 6A). Note that *17BC4* mRNA levels begin to decrease at E17.5 which is consistent with the delimitation of these gene transcripts to nervous tissue.

To determine if the mutagenized gene is present as a single copy and if the gene trap had inserted only once in the genome, Southern blots were performed. The presence of a single band for multiple restriction digests of mouse genomic DNA would indicate that the gene is a single copy and has no close homologs. Figure 6B shows a Southern blot of mouse genomic DNA digested with three different restriction endonucleases and hybridized to a *17BC4*-specific probe. The probe was generated from a 900-bp *Eco*RI fragment of a 2.6-kb *17BC4* cDNA, corresponding to the middle of a putative large open reading frame (data not shown). The probe hybridized to a single band in each of the three lanes, suggesting that *17BC4* is a single copy gene. To determine if the ES cell clones contain only a single gene trap insertion, genomic DNA from each clone was digested with *Eco*RI, blotted, and hybridized with a probe homologous to the 5′ end of β-geo. The results, shown in Fig. 6C, indicate that the 17BC4 cell clone contains a single gene trap insertion.

indicates a chondrifying region of the shoulder girdle. b, limb bud; g, gut; n, neural tube. (C) Transverse section of an E9.5 embryo ridized with a *6BD4* (*jumonji*) probe, generated from the 5′ RACE product. Elevated levels of expression are observed in the ventromedial ions of the neuroepithelium (arrowheads) and in the outflow tract of the heart (arrow). m, mandibular arch. (D) Coronal section of an .5 embryo hybridized with an *8AB2* probe, generated from the 5′ RACE product. Hybridization signal is strong in the neuroepithelia ounding the third ventricle (3) and lateral ventricles (l) of the brain and in the trigeminal ganglia (t). e, eye; o, oral cavity. (E) Parasagittal ion of an E16.5 embryo hybridized with an *8AB2* probe, generated from a 600-bp cDNA clone, which shows signal in the intercostal scles between the ribs (r) and skeletal muscle (arrows) around the shoulder blade (s). f, brown fat. (F) A section serial to that in E hybridized the skeletal muscle-specific probe myf-6. (G) Parasagittal section of the hippocampus of adult brain hybridized with the *17BC4* probe, rated from a 750-bp cDNA. Signal is evident in the granule cell layers of the dentate gyrus (d) and the horn of ammon (a). (H) A section l to that in G hybridized to the *17BC4* sense control probe. c, cortex. Scale bars A–D, G–H = 300 μm, E–F = 600 μm.

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In summary, the technique of *in vitro* preselection has enabled us to identify trapped genes that are expressed in selected cell types *in vitro* and *in vivo* and characterize them on a molecular level to verify their suitability for generating transgenic animals (Fig. 1). We are currently making chimeric mice using ES cell clones that contain probable null alleles of novel, single copy genes that are expressed in a limited number of embryonic tissues.

DISCUSSION

In Vitro Preselection: An Economical Alternative to Standard Gene Trap Screens

Gene trapping is a powerful experimental approach for identifying and studying novel, developmentally regulated genes in the mouse. Gene traps are designed to disrupt normal gene function by randomly inserting a *lacZ* marker gene into transcription units. In previous studies, these trapped genes were initially characterized by the expression patterns of the marker in chimeric embryos. Gene traps that are expressed in intriguing developmental patterns may be selected for further analysis. Two large-scale gene trap screens (Friedrich and Soriano, 1991; Wurst *et al.*, 1995) have shown that the majority of gene trap insertions either are not expressed or are expressed ubiquitously in chimeric embryos during the developmental stages that were assayed; relatively few gene traps exhibited a highly restricted expression pattern, a criterion that usually provides the basis for continued investigation. Thus, gene trap screens are generally conducted on a large scale (i.e., 30–300 chimeric lines; Friedrich and Soriano, 1991; Wurst *et al.*, 1995). These screens involve an investment of labor, time, and expense that may be prohibitive for most investigators. Moreover, many institutions do not have the facilities to maintain large numbers of mouse colonies, and most labs are not equipped to generate chimeric or transgenic animals and must therefore employ the services of a transgenic animal facility.

In this report we describe an alternate approach, which we term *in vitro* preselection, that has several advantages over standard gene trap screens. First, a large number of ES cell clones may be simultaneously differentiated and screened for gene trap expression. One can screen up to 200 clones for tissue-restricted gene trap expression within a few months and eliminate housekeeping genes that are ubiquitously expressed in undifferentiated and differentiated cells. Generating and examining a comparable number of chimeric embryo litters would be a far more expensive and time-consuming endeavor.

Second, *in vitro* preselection reveals how much, if anything, is already known about the trapped gene. If endogenous sequences are not obtained prior to making transgenic mice, considerable time and effort may be invested in generating animals with mutations in previously characterized genes.

Third, *in vitro* preselection identifies which novel genes are redundant or alternately spliced and therefore less likely to result in an overt phenotype in transgenic animals homozygous for the gene trap insertion. For example, many developmentally important genes are members of multigene families that may have redundant functions (e.g., *MyoD* and *myf-5*; Rudnicki *et al.*, 1993). Moreover, gene traps may not impair the function of genes if they have alternately spliced transcripts (such that some mRNA variants do not contain gene trap-specific sequences) or if their transcripts retain much of their endogenous sequences. As we have shown here, these concerns can be addressed by routine molecular analyses. Southern blots indicate if the gene has multiple copies or related genes, and Northern blots reveal how much of the endogenous transcript(s) is replaced by gene trap-specific sequences. The ability to identify probable knockout alleles of single copy genes will prove useful for deciding which ES cell clones are most suitable for generating transgenic animals.

Fourth, *in vitro* preselection greatly reduces the numbers of embryonic and adult mice that must be sacrificed to determine *in vivo* gene trap expression patterns. In standard gene trap screens, chimeric embryos at a series of developmental stages (requiring more than one litter per ES cell clone) are dissected from euthanized mothers, fixed, and stained with X-gal. *In vitro* preselection utilizes wild-type embryo sections, which are hybridized to cRNA probes homologous to the endogenous trapped gene transcripts; thus, sections from a dozen embryos may be used to determine the developmental expression patterns of approximately 50 genes. By this approach, chimeric animals are generated solely for the purpose of obtaining germline transmission of mutagenized genes that are already extensively characterized.

Finally, *in vitro* preselection will yield additional benefits whether or not a gene trap insertion results in an overt mutant phenotype. ES cell and transgenic mouse lines created in this fashion will be of interest for research in murine development, since *lacZ* provides a convenient marker for marking and tracing particular cell lineages. As more studies involve gene trapping, a large number of chromosomes tagged with *lacZ* insertions will eventually be generated. These genetic tags will be of great benefit in future efforts to map the murine genome and to define particular loci.

Widespread Expression of Trapped Genes Does Not Preclude Tissue-Specific Functions

A common pattern observed in standard gene trap screens is early widespread expression of the gene trap (Friedrich and Soriano, 1991; Skarnes *et al.*, 1992; Wurst *et al.*, 1995; this report). For studies in which the ROSAβ-geo gene trap was used (Friedrich and Soriano, 1991; this report), gene trap expression is obligatory in undifferentiated ES cells, since activity of the selected neo^R function requires that the trapped gene is transcriptionally active. However, gene trap constructs in which neo^R and β-gal activities are uncoupled (i.e., *neo*^{R} is regulated by a constitutive promoter) can also exhibit an early widespread β-gal expression pattern that

becomes restricted at later stages (Skarnes *et al.*, 1992; Wurst *et al.*, 1995).

Several explanations may account for this observation. First, genes that are transcribed at high levels only within specific tissues during later stages of development may be transcribed at lower levels in undifferentiated ES cells. For example, the myogenic gene *myf-5*, which is expressed in somitic mesoderm beginning at E8.0, was found to be expressed in undifferentiated ES cells (Miller-Hance *et al.*, 1993). Such genes are thus liable to be detected by *in vitro* preselection. Second, genes may be transcribed ubiquitously but translated only within specific cell types. Sequences contained within the endogenous 3′ UTRs of trapped genes may be involved in their translational repression (for reviews see Richter, 1991; Schäfer *et al.*, 1995).

The ROSAβ-geo construct offers tremendous potential for identifying genes that exhibit tissue-specific functions despite their widespread expression patterns. The insertion of ROSAβ-geo into the *TEF-1* gene, for example, exhibited widespread expression in embryos between the eight-cell stage and stage E12 (Chen *et al.*, 1994). Embryos homozygous for this gene trap exhibit developmental defects specific to the heart and nervous system, resulting in embryonic death between E11 and E12. Similar findings were made by Takeuchi *et al.* (1995), who used a different gene trap construct to identify and clone *jumonji*, which is also expressed in undifferentiated cells. Embryos homozygous for the *jumonji* gene trap allele exhibit specific defects within the developing neural tube and brain and die between E12.5 and E15.5.

In summary, *in vitro* preselection is a powerful alternative approach to *in vivo* gene trap screens that is comparatively efficient and cost effective. By varying the type of gene trap construct used, one may select for genes that are expressed at early stages of development and are subsequently downregulated (e.g., by using the ROSAβ-geo), or one may screen for genes that are activated only after the onset of differentiation (e.g., by using a construct in which neo^R and *lacZ* are transcriptionally uncoupled). By characterizing mutagenized genes prior to making transgenic animals, it is possible to avoid generating transgenic animals having mutations in multicopy genes, ubiquitous housekeeping genes, or genes that have been previously analyzed. These considerations will allow investigators with limited resources to conduct large-scale screens for novel genes expressed in embryonic cell types that can differentiate in cultures of ES cells.

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